DOI: 10.1534/genetics.105.041004

Evolutionary Conservation of *Ceratitis capitata transformer* Gene Function

Attilio Pane,*,[1] Annamaria De Simone,* Giuseppe Saccone* and Catello Polito*,†

**Dipartimento di Genetica, Biologia Generale e Molecolare, Università degli Studi di Napoli "Federico II," 80134 Naples, Italy and †Istituto di Genetica e Biofisica, A. Buzzati-Traverso, CNR 80125 Naples, Italy*

Manuscript received January 22, 2005
Accepted for publication June 24, 2005

ABSTRACT

Transformer functions as a binary switch gene in the sex determination and sexual differentiation of *Drosophila melanogaster* and *Ceratitis capitata*, two insect species that separated nearly 100 million years ago. The TRA protein is required for female differentiation of XX individuals, while XY individuals express smaller, presumably nonfunctional TRA peptides and consequently develop into adult males. In both species, *tra* confers female sexual identity through a well-conserved *double-sex* gene. However, unlike Drosophila *tra*, which is regulated by the upstream *Sex-lethal* gene, Ceratitis *tra* itself is likely to control a feedback loop that ensures the maintenance of the female sexual state. The putative CcTRA protein shares a very low degree of sequence identity with the TRA proteins from Drosophila species. However, in this study we show that a female-specific Ceratitis *Cctra* cDNA encoding the putative full-length CcTRA protein is able to support the female somatic and germline sexual differentiation of *D. melanogaster* XX; *tra* mutant adults. Although highly divergent, CcTRA can functionally substitute for DmTRA and induce the female-specific expression of both *Dmdsx* and *Dmfru* genes. These data demonstrate the unusual plasticity of the TRA protein that retains a conserved function despite the high evolutionary rate. We suggest that *transformer* plays an important role in providing a molecular basis for the variety of sex-determining systems seen among insects.

In the fruit fly *Drosophila melanogaster*, the X:A ratio serves as the primary signal for somatic sex determination. The primary signal acts via *Sex lethal* (*Sxl*), which functions as the binary switch gene that controls sexual differentiation. When the X:A ratio is 1 (2X/2A embryos), *Sxl* is switched "on" and signals female identity, whereas a value of 0.5 (X/2A embryos) turns *Sxl* "off," allowing male development to ensue (Cline and Meyer 1996). During early development, *Sxl* is transiently transcribed from an early promoter (Sxl^{Pe}) in only the XX embryos in response to the primary signal, while at later stages the transcription of *Sxl* is triggered by a maintenance promoter (Sxl^{Pm}) in both males and females (Keyes *et al.* 1992). The early SXL protein establishes a positive feedback loop in the XX embryos by directing the female-specific productive splicing of *Sxl* pre-mRNA transcribed from Sxl^{Pe} (Cline 1984; Bell *et al.* 1991). This autoregulatory mechanism guarantees the memory of the female sexual state throughout the life cycle of the fly. By contrast, in males, transcription from Sxl^{Pe} is not activated and therefore the feedback loop is never initiated. *Sxl* controls the sexual differentiation program through the downstream *transformer* (*tra*) gene. In females, SXL protein directs the splicing of *tra* RNA so that the full-length TRA protein is produced only in females (Sosnowski *et al.* 1989; Inoue *et al.* 1990; Valcárcel *et al.* 1993). In males, the *Sxl* gene is "off"; therefore *tra* splicing is governed by a default mechanism resulting in small nonfunctional TRA peptides (Butler *et al.* 1986; Boggs *et al.* 1987; McKeown *et al.* 1987). TRA subsequently controls the downstream components of the cascade, namely the *double-sex* (*dsx*) and *fruitless* (*fru*) genes. Indeed, it has been shown that TRA is able to direct the female-specific processing of *dsx* and *fru* precursor mRNAs (Baker and Ridge 1980; McKeown *et al.* 1988; Hoshijima *et al.* 1991; Heinrichs *et al.* 1998). This process requires the product of the sex-non-specific *transformer2* (*tra2*) gene and general splicing factors (Amrein *et al.* 1988; Inoue *et al.* 1992). Female-specific mRNAs are produced from *dsx* and *fru* encoding the DSX^F and FRU^F proteins in the presence of the TRA-TRA2 complex. While in males, in the absence of TRA protein, *dsx* and *fru* give rise to the male-specific DSX^M and FRU^M isoforms (Burtis and Baker 1989; Ryner *et al.* 1996). The protein isoforms produced from the *dsx* and *fru* genes are responsible for the development of sex-specific somatic tissues and behavioral traits.

We have recently reported on the molecular cloning of the first *tra* homolog (*Cctra*) in a nondrosophilid insect species, namely the Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Pane *et al.* 2002), which diverged from *D. melanogaster* ~100 million years ago (Beverly and Wilson 1984). The primary signal in Medfly relies on a Y-linked male-determining factor (M-factor) that signals male identity. Consequently, XY embryos develop into adult males, while XX embryos turn into females. The

[1]*Corresponding author:* Molecular Biology Department, Princeton University, Washington Rd., Princeton, NJ 08544.
E-mail: apane@molbio.princeton.edu

Sxl homolog in Medfly (*CcSxl*) does not function as a key switch gene in the sex-determination process (Saccone *et al.* 1998). Instead, a master sex-determining activity in Ceratitis is exerted by the *tra* homolog. Interestingly, *Cctra* shares several key features with both *Dmtra* and *DmSxl*. Similar to *Dmtra*, *Cctra* generates mRNAs encoding the full-length CcTRA protein only in females. Also, *Cctra* seems to act via a conserved Ceratitis *double-sex* (*Ccdsx*) homolog (Saccone *et al.* 2000). Therefore, the *tra*>*dsx* genetic segment seems to be conserved in these species, while the *Sxl*>*tra* one is present in Drosophila, but not in Ceratitis. This genetic architecture is sustained by the fact that *Cctra* displays an autocatalytic function in Ceratitis, similar to that of *DmSxl*, which guarantees the cell memory of the female sexual state. In XY embryos, *Cctra* autoregulation is likely to be inhibited by the Y-linked male-determining factor (M-factor), and male development is launched. In support of this hypothesis, when *Cctra* activity is transiently abolished by RNA interference (RNAi) in XX early embryos, the emerging XX adults express male-specific mRNA variants of *Cctra* and develop as males (Pane *et al.* 2002). The sex choice established by the state of activity of *Cctra* is then transmitted to a downstream *dsx* homolog (*Ccdsx*). This process appears to be very similar to that observed in Drosophila, since *Ccdsx* displays a high degree of sequence and structural identity with *Dmdsx* as well as a similar sex-specific expression (Saccone *et al.* 2002). In agreement with these observations, when *Cctra* is turned by RNAi from the female to the male mode of splicing in XX individuals, the expression pattern of *Ccdsx* changes accordingly (Pane *et al.* 2002).

It has been shown that the *tra* gene undergoes a rapid evolutionary divergence even though it governs a fundamental aspect of fly development (O'Neil and Belote 1992; McAllister and McVean 1999; Kulathinal *et al.* 2003). A low degree of amino acid sequence conservation is consistently revealed when the TRA proteins from several Drosophila species are aligned. For instance, the protein products of *Drosophila virilis* and *D. melanogaster*, which separated 60 MYA, share 36% amino acid sequence identity (O'Neil and Belote 1992). In agreement with the phylogenetic distance between Ceratitis and Drosophila, we found that Ceratitis TRA is 17.9% identical to DmTRA and 18% identical to DvTRA, and it is also more than twice as long (429 aa *vs.* 197 and 199 aa, respectively). Although the evolution of the *tra* sequences has been analyzed in several studies, the functional conservation of this gene has been poorly investigated through *in vivo* experiments. To date, only one report has shown that the *D. virilis tra* can partially supply the *tra*+ function when introduced in *D. melanogaster tra*− mutant strains (O'Neil and Belote 1992).

To test the activity of Ceratitis *tra* in *D. melanogaster*, we expressed a *Cctra* cDNA encoding the full-length CcTRA protein in Drosophila transgenic lines. We show that the *Cctra* transgene is able to promote sexual transformation of Drosophila males into pseudofemales and to rescue the phenotype of XX; *tra* mutant females. Consistent with the phenotypic effect of *Cctra*, endogenous *dsx* and *fru* gene expression are turned from a male to a female mode of splicing in the presence of CcTRA protein. Our results show that despite the minimal conservation, CcTRA is able to recognize DmTRA target sequences and protein partners during development of the fly.

MATERIALS AND METHODS

Molecular biology techniques: Restriction digestion, plasmid construction, blotting, or hybridization were essentially as in Sambrook *et al.* (1989).

Plasmid construction: The *Cctra*F1 cDNA was removed as an *Xba*I/*Xho* fragment from Bluescript vector and subcloned into *Xba*I and *Sal*I-digested pHSS6-hsp-t (Seifert *et al.* 1986), which resulted in pHSS6-hsp-*Cctra*F1-t intermediate plasmid. The cassette containing the *Cctra*F1 cDNA under *D. melanogaster hsp70* promoter (*hsp70*p) and terminator (*hsp70*t) regions was isolated as a *Not*I fragment. This fragment was then cloned into the unique *Not*I site of the pSLfa1180fa shuttle vector (Horn and Wimmer 2000), yielding the pSL-hsp-*Cctra*F1-taf plasmid. The pSLfa1180fa cloning vector contains *Fse*I and *Asc*I restriction sites flanking the multiple cloning site of pSL1180 (Amersham Pharmacia). An *Fse*I/*Asc*I fragment, carrying the *hsp70*pr-*Cctra*F1-*hsp70*t gene cassette, was removed from pSL-hsp-*Cctra*F1-taf and cloned into *Fse*I/*Asc*I-digested *piggyBac*-[3xP3-EGFP] vector (Handler and Harrell 1999). The final transformation vector was named pBac(3G)-hs-*Cctra*F1 (Figure 1A).

Germline transformation: Germline transformation experiments were performed as described by Rubin and Spradling (1982). Preblastoderm embryos of *D. melanogaster* were injected with 500 ng/μl of pBac(3G)-hs-*Cctra*F1 and 150 ng/μl of the helper phsp-pBac vector (Handler and Harrell 1999). The phenotypic marker carried by the pBac(3G)-hs-*Cctra*F1 plasmid is a cDNA encoding the enhanced green fluorescent protein (EGFP) under the control of the 3xP3 artificial promoter (Horn and Wimmer 2000). This promoter drives the expression of the EGFP in the larval central nervous system (CNS) and in the eyes of Drosophila adults (Horn and Wimmer 2000). Transgenic individuals were detected in the G_1 progeny for the presence of green fluorescent eyes. All EGFP fluorescence observations were conducted employing the LEICA MZ FLIII stereomicroscope and the GFP2 (GFP Plus) filter set (excitation filter, 480/40 nm; barrier filter, 510 nm).

We collected 32 transgenic individuals showing the expected eye fluorescence, which were generated by four F_0 single crosses. Genomic Southern blots on three lines, 9a, 9i, and 33h, revealed that each line is characterized by a single insertion of the pBac(3G)-hs-*Cctra*F1 transposable element and confirmed that the transgenic lines were derived from independent integration events (data not shown). Line 9a had the transposable element inserted on chromosome II and could be established by crossing transgenic individuals with the double-balanced marker strain *yw; CyO/Sco; TM3*. Other lines described in this article were kept unbalanced. Line 33h had a single insertion of the pBac(3G)-hs-*Cctra*F1 on the X chromosome. Lines 9a and 33h were chosen as the best representatives of the different phenotypic classes, since they show, respectively, the weakest and the strongest penetrance of the transgene at 25°.

To induce the expression of the cDNA *Cctra*F1 from the *Hsp70* promoter, flies were exposed to a daily heat-shock regimen at 37° for 1 hr from embryonic stages until adulthood. PCR-based karyotipic analysis and genetic markers (Bs

TABLE 1

Oligonucleotides used in this study

Gene	Oligonucleotides	Location
Tra	199+ (5′-GGCACATTGCAAGGTGCAAG-3′)	Exon 1
	1081− (5′-GCTGGCGGTATCTGTAGGG-3′)	Exon 3
Dsx	Dsx 1 (5′-CCGCTATCCTTGGGAGCT-3′)	Exon 3 (non-sex specific)
	Dsx 2 (5′-TTGAGATTGGCTTGTATGCC-3′)	Exon 4 (female specific)
	Dsx 3 (5′-GACCATTGTGGGTGAGGC-3′)	Exon 5 (male specific)
Fru	Fru 1 (5′-GGAAATCGTCTCGAAGTAGGAC-3′)	Exon 3 (non-sex specific)
	Fru 2 (5′-TGCATTACGCGGCCTTGGAC-3′)	Exon 2 (male specific)
	Fru 3 (5′GAATTCGAGGACGTGTGACGAT-3′)	Exon 2 (female specific)

allele on the Y chromosome) were used to distinguish XX females from XY pseudofemales recovered after heat-shock treatment (data not shown).

RT-PCR: Total RNA was extracted from adult Drosophila individuals as described elsewhere (Andres and Thummel 1994). Oligo(dT)-primed cDNA was made from DNAase-treated total RNA of adult flies using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen, San Diego). RT-PCR experiments to investigate the state of activity of Drosophila sex-determining genes were performed with the primers reported in Table 1.

Cycling conditions were denaturation at 94° for 5 min, followed by 35 cycles of 94° for 30 sec, annealing at 58° (for *dsx*), 62° (for *tra*), and 64° (for *fru*) for 30 sec, and extension at 72° for 1 min, with a final 5-min extension at 72°.

RT-PCR products were transferred to Hybond NX nylon membranes (Amersham Pharmacia) and blotted with a ^{32}P-radiolabeled probe corresponding to a sex-non-specific region of *fruitless*. The clone containing the *fru* probe was kindly provided by Stephen Goodwin.

Fly strains: Transgenic strains were produced by microinjection of DNA constructs into a *D. melanogaster white-eye* (w^{1118}) strain. *tra* mutant lines (*BsY; tra, e, ca/TM3, sb, ser*) and [*BsY; Df(3L)E34, st, tra, e/TM3, sb, ser*] were kindly provided by Daniel Bopp. These lines accumulated recessive lethal alleles on chromosome III. Hence, hs-*Cctra*F1 activity was analyzed in a *trans*-heterozygous *tra* mutant background. *tra2* mutant strains *DfTRIX/CyO* and *tra2B/CyO* were kindly provided by William Mattox.

RESULTS

hs-*Cctra*F1 directs female development of XY transgenic flies: The evolutionary conservation of TRA function was investigated by testing the feminizing activity of the *C. capitata* TRA protein when expressed in the distantly related species *D. melanogaster*. To this aim, we generated transgenic strains of Drosophila that carry the Ceratitis female *Cctra*F1 cDNA, encoding the full-length putative CcTRA protein (Pane *et al.* 2002) under the control of the Drosophila *hsp70* promoter (Figure 1A). Transgenic lines were grouped into phenotypic classes according to the severity of perturbation in male development at 25°. In the weak class (Figure 1B), the majority of transgenic males and females had no obvious sex transformation when raised at 25°. They had normal external morphology and appeared fully viable and

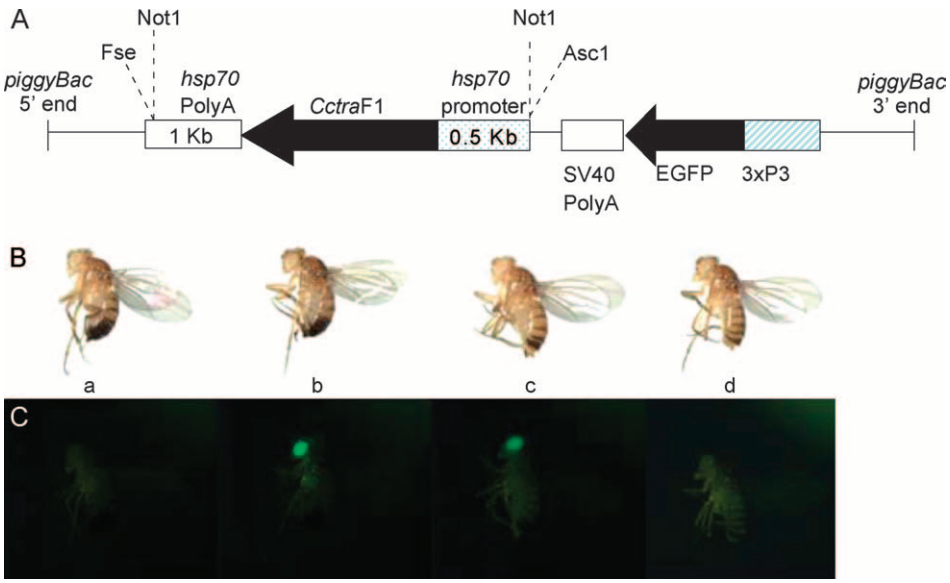

Figure 1.—(A) Structure of the pBac-[3xP3-EGFP]-*Cctra*F1 transformation vector. (B) Phenotypic analysis of hs-*Cctra*F1 transgenic lines at 25°. Wild-type males and females are reported in a and d, respectively. XY individuals from the weak line 9a develop as normal males (b), while XY adults of the strong line 33h are phenotypic females (c) at 25°. (C) EGFP fluorescence observation through the GFP2 filter set.

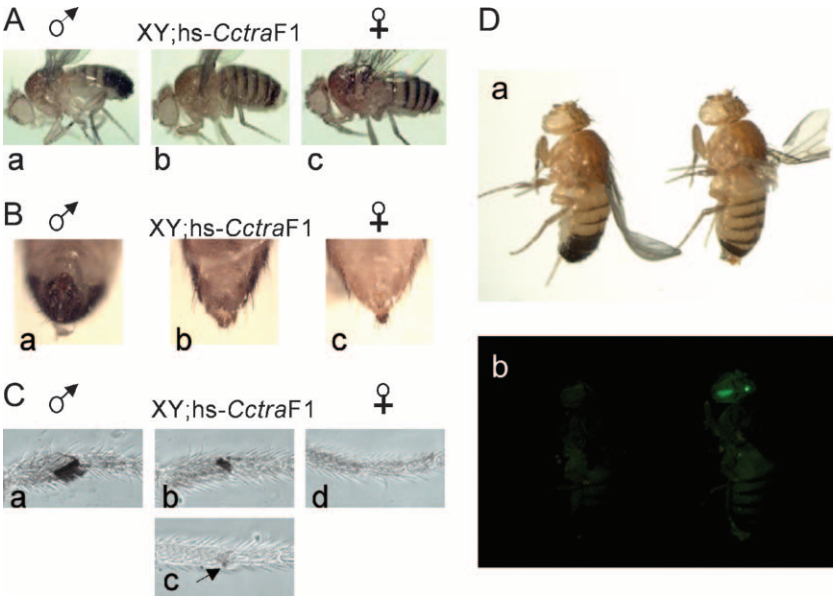

FIGURE 2.—Phenotypic analysis of XY; hs-*CctraF1* adults. Heat-shock induction of the hs-*Cctra*F1 transgene drives the development of females from otherwise male XY embryos. (A) Abdominal pigmentation. (a) *w* males show fully pigmented fifth and sixth tergites; (b) XY; hs-*Cctra*F1 individuals show female-like abdomens after heat-shock induction of the hs-*Cctra*F1 transgene; (c) in the *w* females, pigmentation is restricted to a narrow stripe in each tergite. (B) Morphology of the external genitalia. Typical genital apparatus of *w* males (a) and females (c) are shown. (b) XY; hs-*CctraF1* pseudofemales recovered after heat-shock treatment display nearly normal female external genitalia. (C) Analysis of sex-comb bristles. (a) *w* male sex-comb bristles; (b) forelegs of XY; hs-*Cctra*F1 intersexes that were not subjected to heat-shock regimen show a marked reduction of the sex-comb bristles (three to four bristles instead of the typical seven); (c) heat-shock induction of the hs-*Cctra*F1 transgene in XY; hs-*Cctra*F1 individuals prevents formation of the sex combs; (d) forelegs of *w* females lack sex-comb bristles. (D) Analysis of the body size of XY; hs-*Cctra*F1 pseudofemales. (a) Male individuals of the strong line 33h develop as pseudofemales at 25°, but they are similar in size to wild-type males. (b) EGFP fluorescence observation through the GFP2 filter set.

fertile. However, a small percentage (2%) of males showed male intersexual phenotypes at 25°. These males had reduced pigmentation of the abdomen and incomplete development of sex-comb bristles. Also, their external genitalia exhibited a variable extent of feminization. In the intermediate class, transgenic males displayed a variety of phenotypes, ranging from male to female-like external morphology. In this class, a high percentage of animals (30%) were intersexes and revealed a phenotype similar to that observed in the weak lines. Finally, in the strong class (Figure 1B), transgenic males were always strongly feminized. They exhibited female-like reduced abdominal pigmentation and no sex combs on the forelegs. In addition, external genitalia were made up of both male and female tissues.

Males from all three classes of lines examined were strongly transformed toward femaleness when exposed to a daily heat-shock regimen (Figure 2). Indeed, these individuals developed female-like abdominal pigmentation (Figure 2A) and apparently normal female-like external genitalia (Figure 2B). Furthermore, sex-comb bristles on their forelegs were reduced or absent (Figure 2C). However, pseudofemales from all the classes were sterile most likely because their germline failed to undergo a complete sex transformation. Males of line 9a with one copy of the transgene did not undergo a complete somatic sex transformation and developed as intersexes. XX; hs-*Cctra*F1 transgenic females neither were affected by the basal activity of the transgene at 25° nor displayed sexually altered somatic tissues after heat-shock treatment.

In Drosophila, one of the most evident dimorphic traits is represented by the body size, with males being smaller than females. It has been previously reported that this character relies on the state of activity of *Sxl* and is independent of *Dmtra* (CLINE 1984; WATERBURY *et al.* 2000). In agreement with these previous reports, we observed that hs-*Cctra*F1 pseudofemales express male mRNA variants of *Sxl* and develop a body size similar to that of wild-type males (Figure 2D).

***dsx* and *fru* splicing is altered after hs-*Cctra*F1 induction:** In *D. melanogaster*, the presence of a functional TRA protein triggers the expression of female-specific protein isoforms from *dsx* and *fru*, which in turn direct female differentiation. Conversely, when TRA is absent, a default mechanism gives rise to male-specific proteins from *dsx* and *fru* and male development ensues. We have shown that ectopic expression of hs-*Cctra*F1 in Drosophila XY transgenic flies is sufficient to trigger feminization of somatic tissues. Hence we thought that the CcTRA protein, encoded by the transgene, is able to mimic the function of DmTRA and to promote the expression of *dsx* and *fru* female splice variants. To test whether hs-*Cctra*F1 causes a shift in *dsx* and *fru* splicing patterns from a male to a female mode, RT-PCR experiments were performed on RNA extracted from hs-*Cctra*F1 adults (Figure 3). In Drosophila, *dsx* message is spliced in a sex-specific manner. In the case of females, the mature *dsx* transcript consists of both exons 3 and 4, whereas in males an alternative splicing event leads to elimination of exon 4. Therefore, mature transcripts contain exon 3 fused directly to exon 5. The expression pattern of *dsx* was determined with a common forward primer located in exon 3, and female- and male-specific reverse primers located, respectively, in exons 4 and 5 (Figure 3A). The set of primers that we designed allowed us to visualize the sex-specific splice variants of *dsx* in wild type as well as in transgenic males and females (Figure 3A). A slowly migrating band is detected by

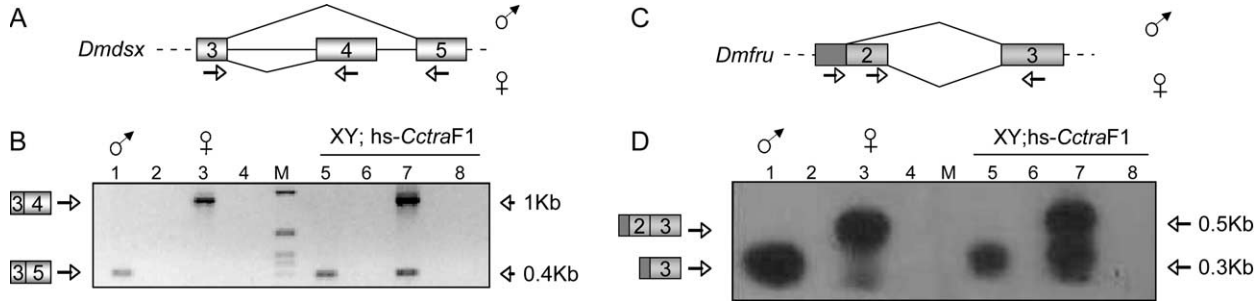

FIGURE 3.—Expression of the *Dmdsx* and *Dmfru* genes in adult XY; hs-*CctraF1* pseudofemales. (A) Position of the synthetic oligonucleotides within the *Dmdsx* gene (arrows). (B) RT-PCR reactions with *Dmdsx*-specific primers on *w* males (lane 1) and females (lane 3) generate 0.4- and 1-kb fragments, respectively. RT-PCR on XY; hs-*Cctra*F1 untreated adults produce a 0.4-kb fragment only (lane 5). Treated XY; hs-*Cctra*F1 pseudofemales generate both 0.4- and 1-kb amplification products (lane 7). (C) Schematic showing the position of the synthetic oligonucleotides in the *fru* gene (arrows). (D) RT-PCR with *fru*-specific primers generate 0.3- and 0.5-kb amplification products in wild-type males (lane 1) and females (lane 3), respectively. Only the male-specific band can be detected in untreated XY; hs-*Cctra*F1 males (lane 5), while both the amplification fragments are visible in XY; hs-*Cctra*F1 pseudofemales (lane 7). Even-numbered lanes show negative RT-PCR controls. A molecular weight marker is shown in lane M.

RT-PCR in the wild-type female sample as compared to the male lane (Figure 3B, lanes 1 and 3). RT-PCR on XY; hs-*Cctra*F1 adults produce a male-specific amplification product in the absence of heat-shock treatment (Figure 3B, lane 5). However, upon heat shock, an additional band is readily detectable in the XY; hs-*Cctra*F1 sample lane. The size of this band corresponds to the female-specific amplification product (Figure 3B, compare lane 1 to lane 7).

Another known target of *tra* in Drosophila is the *fruitless* gene. It was previously shown that *fru* expression is restricted to a small number of neurons in the CNS to regulate sex-specific behavior (VILLELLA *et al.* 1997). The precursor mRNAs transcribed from the *fru* locus undergo alternative splicing and generate differential mature transcripts in females and males. Consistently, in females the Tra-Tra2 complex interacts with the "Tra-Tra2-binding sites" in *fru* exon 2 and engineers the inclusion of this exon in the mature transcripts (HEINRICHS *et al.* 1998). Conversely, in males, in the absence of TRA protein, the spliceosome recognizes an upstream 5′ splice site, resulting in exclusion of a portion of exon 2 from the mature *fru* mRNAs.

We determined the expression pattern of *fru* by RT-PCR using forward primers located in exon 2, sex-non-specific and male-specific regions, and a reverse primer in exon 3 (Figure 3C). The RT-PCR reaction generates a male product with lower molecular weight with respect to the female amplification fragment (Figure 3D, lanes 1–3). In the absence of heat-shock induction, XY; hs-*Cctra*F1 individuals generate only the male-specific amplification product (Figure 3D, lane 5). By contrast, male- and female-specific bands are detectable in the XY; hs-*Cctra*F1 pseudofemales (Figure 3D, lane 7).

Does *Cctra* regulate endogenous *Dmtra* expression? We have shown that hs-*Cctra*F1 can regulate female-specific processing of *dsx* and *fru* precursor transcripts and thereby promote female-like differentiation of Drosophila XY individuals. However, these data, *a priori*, do not rule out the possibility that hs-*Cctra*F1 actually directs the splicing of *Dmtra*, rather than acting directly on *dsx* and *fru*. Indeed, in Ceratitis, *Cctra* is thought to regulate its own expression through a positive feedback loop (PANE *et al.* 2002). The model proposes that CcTRA protein autoregulates by binding to the "Tra-Tra2-binding sites" in *Cctra* intron 1 and directs the exclusion of this intron from the female splice variants. This mechanism in turn sustains the continuous expression of CcTRA protein needed for remembering the female identity. To determine whether CcTRA might direct the female splicing of *Dmtra*, RT-PCR experiments were carried out using primers that allow detection of both the sex-non-specific and the female-specific transcripts of *Dmtra* in the transgenic lines (Figure 4A). As reported previously, the wild-type male samples show only the sex-non-specific transcript (Figure 4B, lane 1), while female samples show both the female-specific and the sex-non-specific transcripts (Figure 4B, lane 3). Only the sex-non-specific class of *Dmtra* transcripts are detectable in XY; hs-*Cctra*F1 individuals grown at 25° (Figure 4B, lane 5). Furthermore, the expression pattern of *Dmtra* is not altered even after heat-shock induction of the hs-*Cctra*F1 transgene (Figure 4B, lane 7). We therefore conclude that *Dmtra* is not a target for the hs-*Cctra*F1 transgene and that *Cctra* must regulate alternative splicing of *dsx* and *fru* directly.

Analysis of hs-*Cctra*F1 activity in a Drosophila *tra* mutant background: Since XY animals are morphologically feminized under the control of transgene-derived CcTRA, we wished to determine the extent to which gonad-genitalia formation is influenced by the presence of the hs-*Cctra*F1 transgene. The feminizing activity of hs-*Cctra*F1 during germline development was analyzed by crossing the strong transgenic lines with Drosophila *Dmtra* mutant strains. Similar to XY; hs-*Cctra*F1 flies, XY; hs-*Cctra*F1; *tra* individuals are also transformed into

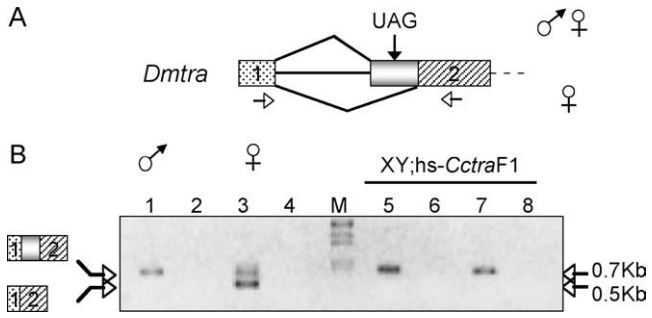

FIGURE 4.—Analysis of the *Dmtra* splicing pattern in adult transgenic individuals. (A) Schematic of synthetic primers in the *Dmtra* gene (arrows). (B) RT-PCR on *w* males (lane 1) detects a 0.7-kb fragment corresponding to the sex-nonspecific *Dmtra* mRNA. In *w* females (lane 3), an additional 0.5-kb PCR product can be observed, which corresponds to a portion of the female-specific transcript. The same primers were used to investigate *Dmtra* expression patterns in untreated or treated XY; hs-*Cctra*F1 individuals (lanes 5 and 7, respectively). Only a 0.7-kb band is visible in these lanes, indicating that the *Dmtra* gene is expressed in the male mode in XY; hs-*Cctra*F1 adults. Expression of the CcTRA protein from the transgene does not alter the expression of endogenous *Dmtra*. Even-numbered lanes show RT-PCR negative controls. A molecular weight marker is shown in lane M.

somatic females at 25° since the hs-*Cctra*F1 transgene does not act through the endogenous *Dmtra*. However, analysis of internal genitalia revealed that these individuals either have malformed testes (Figure 5c) or lack internal genitalia. After heat-shock treatment, a higher percentage of individuals apparently lack germline tissues, but ovarian-like tissues are never formed. A similar phenotype is observed when XX; hs-*Cctra*F1; *tra* females grown at 25° are dissected. However, the ovarian morphology of germline tissues in XX; hs-*Cctra*F1; *tra* females can be rescued after heat induction of the transgene. Indeed, reinforcement of the transgene activity by heat-shock exposure results in the development of rudimentary or even almost-normal ovaries (Figure 5d). Despite the fact that *Cctra* can support female differentiation of both somatic and germline tissues in XX; *tra* individuals, these animals fail to lay eggs, thus suggesting that the rescue of the female phenotype is, however, not complete.

Activity of the hs-*Cctra*F1 transgene is dependent upon a functional endogenous *tra2* gene: In *D. melanogaster*, TRA protein along with its protein partner TRA2 controls all aspects of female-specific differentiation. Therefore, we decided to address if the hs-*Cctra*F1 transgene requires the activity of the endogenous *tra2* gene to promote female differentiation. Indeed, it was shown that DmTRA must assemble into a complex containing DmTRA2 and serine-arginine (SR) splicing factors to direct female expression of *dsx* and *fru* genes. Accordingly, XX flies carrying a nonfunctional *tra2* fail to execute the female program and develop into sterile males (FUJIHARA *et al.* 1978; BAKER and RIDGE 1980). To assess whether CcTRA also requires a functional *Dmtra2* gene, we tested for genetic interaction between hs-*Cctra*F1 and *tra2*. To this end we introduced the hs-*Cctra*F1 transgene into a *tra2* mutant background. Two different *tra2* alleles were used in this experiment, namely $tra2^{B}$ and *DfTRIX* (WATERBURY *et al.* 2000). The chromosomes carrying these alleles accumulated recessive lethal mutations and cannot be kept in a homozygous state. Therefore, we performed the test by generating $tra2^{B}$/*DfTRIX trans*-heterozygous flies, which lack a functional TRA2 protein and develop as adult pseudomales. We found that both strong and intermediate hs-*Cctra*F1 transgenes were unable to direct female differentiation of XY; $tra2^{B}$/*DfTRIX* individuals at 25°. Nor could they rescue the *tra2* mutant phenotype of the XX mutant flies. In addition, both XX and XY; hs-*Cctra*F1; $tra2^{B}$/*DfTRIX* flies showed only male somatic and germline tissues after heat-shock treatment. We conclude that the putative CcTRA protein, produced by hs-*Cctra*F1, requires a functional endogenous TRA2 protein to direct female development of Drosophila transgenic flies.

DISCUSSION

The *transformer* gene plays a fundamental role in the sex determination of *D. melanogaster*. It is responsible for the transduction of the initial sex choice, established by the X/A counting mechanism, to downstream effectors that ultimately control the development of sex-specific traits. Despite its key role in the development of the fly, *tra* reveals an unusual evolutionary rate even when comparison is restricted to Drosophila species. The evolutionary divergence of this gene becomes even more apparent when a non-drosophilid species, namely *C. capitata*, is considered (PANE *et al.* 2002). However, we show here that CcTRA can support not only the somatic, but also the female germline development of Drosophila transgenic strains. Our results demonstrate that CcTRA can substitute for DmTRA during Drosophila sex determination, despite the fact that these proteins

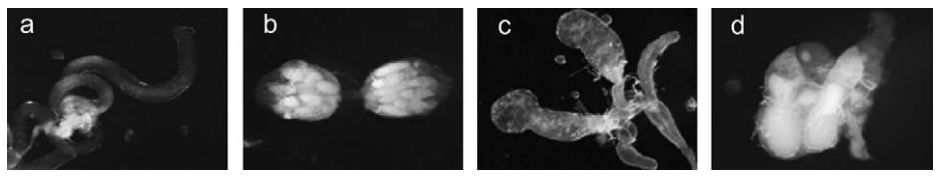

FIGURE 5.—(A) Internal genitalia of hs-*Cctra*F1 adults after heat-shock treatment. (a) Wild-type testes. (b) Wild-type ovaries. (c) Induction of the hs-*Cctra*F1 transgene induces the development of malformed testes in XY; hs-*Cctra*F1 pseudofemales. (d) Ovarian development can be rescued in XX; hs-*Cctra*F1; *tra* individuals after heat-shock treatment.

share only 17.9% sequence identity and are significantly different in size (429 aa *vs.* 197 aa, respectively).

***Cctra* can replace *Dmtra* in the sex determination of Drosophila:** A comparison of Ceratitis TRA with homologous proteins from drosophilid species highlighted only a few short regions of identity (Pane *et al.* 2002). All of the TRA proteins are characterized by a variable number of interspersed serine/arginine dipeptides and a proline/valine motif in the C-terminal domain. In addition, a unique feature shared by these molecules is an 11-amino-acid stretch with a PYF(A/I)DE core sequence, whose function is still unknown. In Ceratitis, however, TRA does not contain the MDMDSS motif at the very N-terminal region, which is present in all of the drosophilids. In this study, we demonstrate that the function of the TRA protein in Ceratitis and Drosophila is conserved despite the high degree of sequence divergence. Consistently, the expression of an hs-*Cctra*F1 transgene, encoding the full-length CcTRA protein, in Drosophila transgenic lines drives the female somatic sexual differentiation of otherwise male individuals. Sex transformation affects all somatic tissues and occurs in the strong transgenic lines even in the absence of heat-shock treatment. A nearly complete somatic sex transformation can also be induced in weak lines after heat-shock exposure, indicating its dependence upon the activity of the hs-*Cctra*F1 transgene. Given the high degree of sequence divergence between CcTRA and DmTRA, it was surprising to discover that hs-*Cctra*F1 can also support germline female differentiation. Indeed, we observe that the expression of a strong hs-*Cctra*F1 transgene can rescue the ovarian morphology in XX; *tra* mutant flies. These flies develop apparently normal ovaries, but upon close inspection, they appear to be sterile and fail to lay eggs. We also observed that ovarian tissues cannot be induced in XY and XY; *tra* individuals. This result is rather unexpected, given the positive effect of heat shock on rescue of XX; *tra* mutants. However, it was previously shown that expression of an hs-*Dmtra*F transgene is not able to feminize the germline of Drosophila XY individuals (McKeown *et al.* 1988). The absence of ovarian tissues in XY flies might be due to the fact that XY germ cells appear to be less responsive to feminizing signals than XX germ cells (Waterbury *et al.* 2000).

In Drosophila, the sexual identity of the developing individual is controlled by *tra* via the downstream *dsx* and *fru* genes. In the XX individuals the TRA protein controls the female-specific splicing of the *dsx* and *fru* pre-mRNAs, which leads to the production of DSX and FRU female protein isoforms. By contrast, in males TRA is not expressed and therefore *dsx* and *fru* are spliced in the default male mode. This event leads to the production of the male-specific DSX and FRU proteins. The *dsx* gene is responsible mainly for the sexual dimorphism of the somatic traits, while *fru* has been shown to control all aspects of male courtship behavior (Villella *et al.* 1997). In agreement with the sex transformation of Drosophila hs-*Cctra*F1 transgenic flies, the endogenous *dsx* and *fru* genes are expressed in the female mode in the resulting XY pseudofemales. Our data strongly suggest that CcTRA directs the female-specific processing of *dsx* and *fru* using a mechanism similar to that of DmTRA. In Drosophila, the TRA protein acts in conjunction with TRA2, a sex-non-specific RNA-binding protein, to trigger female development. TRA and TRA2 assemble into a complex with SR-type splicing factors and recognize short repeats of 13 nucleotides known as "TRA-TRA2 binding sites," located in *dsx* exon 4 and *fru* exon 2 (Hedley and Maniatis 1991; Heinrichs *et al.* 1998). When the TRA-TRA2 complex binds these *cis*-regulatory elements, it reinforces the usage of suboptimal splicing sites in the *dsx* and *fru* primary transcripts and promotes female-specific splicing events (Tian and Maniatis 1993). Accordingly, XX; *tra2* mutants that lack a functional TRA2 protein fail to activate the female processing of *dsx* and *fru* and develop as adult pseudomales. Similar to DmTRA, CcTRA feminizing activity is also abolished in Drosophila *tra2*− flies, thus suggesting that CcTRA requires a functional endogenous *tra2* gene. We conclude that CcTRA is able to substitute for DmTRA and interact with the endogenous TRA2 protein. The CcTRA-TRA2 complex maintains the specificity for the "TRA-TRA2 binding sites" and the ability to direct the proper female-specific splicing of *dsx* and *fru*.

Molecular mechanisms for *Cctra* and *Ccdsx* regulation in Ceratitis: One important finding of this study is that CcTRA is able to "recognize" the TRA-TRA2-binding sites *in vivo*, although they are located in entirely divergent contexts, namely the *Dmfru* and *Dmdsx* genes of Drosophila. It is therefore tempting to speculate that the TRA-TRA2-binding sites are also target sequences for CcTRA activity in Ceratitis. In this species, TRA-TRA2 elements are present in exon 4 of the *dsx* homolog (*Ccdsx*) and in intron 1 of the *Cctra* gene. *Ccdsx* reveals a significant structural and sequence identity when compared to *Dmdsx* and shows a sex-specific expression pattern (Saccone *et al.* 2000, 2002; Pane *et al.* 2002). The distribution of the *cis* elements in the *Ccdsx* gene is also similar to that of *Dmdsx*, since they are located in exon 4, which is sex-specifically regulated in both Ceratitis and Drosophila (Saccone *et al.* 2000). Given that CcTRA can promote the proper female splicing of *Dmdsx*, it is conceivable that it also controls *Ccdsx* expression using a similar mechanism. In Ceratitis females, CcTRA is likely to bind the *cis* elements in *Ccdsx* exon 4 and to promote the fusion of exon 3 to exon 4. The resulting female mature transcripts encode the CcDSX^{F} protein. By contrast, in males, where the CcTRA protein is absent, exon 4 is not included in the mature mRNA, with exon 3 being fused directly to exon 5. The mature mRNAs generated in males encode the CcDSX^{M} isoform. Consistent with this model, when the *Cctra* gene is turned off by RNAi in XX individuals, *Ccdsx* expression

pattern is switched from the female to the male mode of splicing (Pane *et al.* 2002). As in Drosophila, the CcDSX isoforms are likely to control the development of sexually dimorphic traits in Ceratitis.

We have shown that putative TRA-TRA2 elements are also surprisingly contained in intron 1 of the *Cctra* gene (Pane *et al.* 2002). This observation pointed to a role for the CcTRA protein in the processing of *Cctra* precursor mRNA. In Ceratitis, *Cctra* is sex-specifically expressed through post-transcriptional alternative splicing events. In females, intron 1 is removed from the primary transcript and mature mRNAs that encode the full-length CcTRA protein are produced. Differently, mature mRNAs generated in males retain portions of the intron 1 (*i.e.*, male-specific exons), which contain stop codons and thus prematurely interrupt the translation of the CcTRA protein. We have reported that the female splicing of the *Cctra* primary transcripts is dependent upon a functional *Cctra* gene. When *Cctra* is switched off by RNAi in early embryos, the emerging XX adults are males and express male variants from *Cctra*. These observations lead to the hypothesis that, in Ceratitis females, *Cctra* controls its own expression by means of a positive feedback loop. The results reported here further support this hypothesis and suggest that, in females, the CcTRA protein might bind the TRA-TRA2-binding sites in the *Cctra* pre-mRNA and promote female splicing events. The binding of CcTRA to the *cis* elements might prevent the usage of male splicing sites, thus leading the splicing machinery to use the criptic female sites. Consequently, intron 1 is removed from *Cctra* precursor transcripts to produce the female mature mRNAs. An alternative possibility is represented by an activation mechanism in which CcTRA would enforce the usage of female splice sites. This model stems from the observation that the TRA-TRA2 elements are located mainly within the male-specific exons and therefore are included in male mature mRNAs (Pane *et al.* 2002; A. Pane, unpublished results). It is possible that, in females, "male" transcripts are produced by the default mechanism and might behave as splicing intermediates and substrates for CcTRA activity. In this case, the binding of the CcTRA protein to the *cis*-regulatory elements would favor the use of the female splice sites and promote the removal of the male-specific exons. Both the repression and the activation mechanisms that we propose would involve a new property for the TRA proteins as well as an intronic function for the TRA-TRA2 elements, which has not been described before. In females, *Cctra* mature mRNAs have a long open reading frame and represent the source of CcTRA protein to keep the feedback loop active and guarantee the memory of the female sexual state. In males, the M-factor is likely to impair the positive feedback loop at early stages, thus promoting the male developmental program.

Interestingly, CcTRA activity in the Drosophila transgenic lines is dependent upon a functional endogenous *Dmtra2* gene. CcTRA is not able to direct female splicing of *dsx* and *fru* pre-mRNAs in Drosophila when the DmTRA2 protein is absent. We believe that, in Ceratitis also, female development involves the cooperation between CcTRA and a putative TRA2 homolog (CcTRA2), which is yet to be identified. Several observations further support this hypothesis. *tra2* appears to be highly conserved in evolution and *tra2* homologs were described even in humans (Dauwalder *et al.* 1996). Recently, a *tra2* homolog was identified in the housefly *Musca domestica*, which diverged from Drosophila ~100 million years ago. Transient depletion of the *tra2* function in Musca by RNAi triggers the sexual transformation of XX embryos, which normally become females, toward maleness (Dübendorfer *et al.* 2002). These observations all point to the existence of a conserved *tra2* homolog in Medfly as strongly suggested by the sequence conservation of Tra/Tra-2-binding sites observed in the Ceratitis *dsx* homolog (Saccone *et al.* 2000). The CcTRA2 protein might interact with CcTRA to control both the female-specific splicing of *Ccdsx* and the positive feedback loop established by the *Cctra* gene.

Evolution of sex-determining networks: A wide variety of sex-determination systems can be observed in nature, even when the investigation is restricted to small taxonomic groups (reviewed in Marin and Baker 1998; Schutt and Nothiger 2000; Saccone *et al.* 2002). In the dipteran insects, for instance, some species use a male-determining factor on the Y chromosome (Ceratitis, Musca), while others use a single autosomal factor (Megaselia, Culex). Yet, in other species, the primary signal is given by chromosome balance (Drosophila, Sciara) or maternal effects (Chrysomya). A growing body of evidence suggests that, despite the striking diversity of primary systems, the sex choice is translated to terminal differentiation genes via a short hierarchy of control elements. It has been proposed that these genetic hierarchies have evolved in a "bottom-up" fashion (Wilkins 1995), with new upstream regulators being recruited, in the course of evolution, to control ancient downstream elements. Therefore, the bottom-most components of the cascades are expected to be conserved across species, while upstream players can vary. Genetic dissection of the sex-determination process in a number of model organisms has so far supported this model. Homologs of the *dsx* gene, which is the last regulatory element in the cascade, appear to be conserved not only in flies (Shearman and Frommer 1998; Kuhn *et al.* 2000; Ohbayashi *et al.* 2001; Hediger *et al.* 2004), but also in worms and mammals (Raymond *et al.* 1998), while upstream regulators, such as *Sxl*, are not shared (Meise *et al.* 1998; Saccone *et al.* 1998; Serna *et al.* 2004). In this scenario, the *tra* gene represents a key element in gaining insight into the molecular mechanisms underlying the evolution of the sex-determining networks. To date, *tra* homologs have been described only in the drosophilids and in the distantly related

species *C. capitata*. In both Drosophila and Ceratitis, *tra* determines the sexual fate of the developing individual by regulating the expression of the bifunctional *dsx* gene. In this study, we show that the CcTRA protein of Medfly can replace DmTRA in Drosophila sex determination and direct female expression of the endogenous *dsx* and *fru*, despite the high degree of evolutionary divergence. In agreement with previous reports (O'Neil and Belote 1992; McAllister and McVean 1999; Kulathinal *et al.* 2003), we demonstrate that wide rearrangements can occur in both the coding and noncoding region of *tra*, without affecting the sex-determining function of the gene. Our results support the idea that divergent *tra* homologs might control sex determination in yet other dipteran species, where a *dsx* gene has been identified (Pane *et al.* 2002; Saccone *et al.* 2002). Consistent with this hypothesis, a locus encoding a TRA-like protein, named *complementary sex determiner* (*csd*), was discovered to be the primary signal in sex determination of the honeybee *Apis mellifera* (Beye *et al.* 2003; Beye 2004). Although the hymenopteran diverged from the dipteran insects ~270 MYA, Apis retains a conserved *dsx* homolog, thus suggesting that the *tra/csd>dsx* genetic segment exists in the honeybee, similar to the Medfly and Drosophila (Beye 2004). These species, however, evolved different systems to regulate the sex-specific expression of *tra/csd*. In Drosophila, *tra* occupies an intermediate position in the hierarchy and is regulated by the upstream *Sxl* gene. By contrast, in Ceratitis, *tra* is the master switch gene that controls its own expression by means of a positive feedback loop. Finally, in Apis, *csd* encodes the primary signal and has no further upstream regulatory elements. It is likely that the unusual plasticity allowed *tra* to maintain the sex-determining activity through the *dsx* gene, while new upstream regulatory mechanisms were developed. An expectation of this hypothesis is that conserved *tra>dsx* modules might control sex determination in many other insects, which may have explored alternative systems to regulate its state of activity. We believe that the flexibility of *tra* and the recruitment of different players to control its expression provided a genetic basis for the evolution of different primary sex-determining signals in the dipteran as well as in more distantly related species.

We are grateful to Gerish Deshpande, Patricia Graham, Paul Schedl, Trudi Schupbach, and Andrew Swan for their critical reading of the manuscript and for stimulating discussions. We thank Daniel Bopp, Stephen Godwin, and William Mattox for kindly providing fly strains and Ennio Giordano and Rosaria Rendina for helpful suggestions. We also thank Rosaria Terracciano and Giovanni Manno for technical assistance. This work was supported by Ministero della Ricerca Scientifica e Tecnologica (PRIN 2001-03; FIRB 2003-05).

LITERATURE CITED

Amrein, H., M. Gorman and R. Nothiger, 1988 The sex-determining gene *tra-2* of Drosophila encodes a putative RNA binding protein. Cell **55:** 1025–1035.

Andres, A. J., and C. S. Thummel, 1994 *Drosophila melanogaster: Practical Uses in Cell and Molecular Biology*, pp. 570–573. Academic Press, San Diego.

Baker, B. S., and K. A. Ridge, 1980 Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. Genetics **94:** 383–423.

Bell, L. R., J. I. Horabin, P. Schedl and T. W. Cline, 1991 Positive autoregulation of *Sex-lethal* by alternative splicing maintains the female determined state in Drosophila. Cell **65:** 229–239.

Beverley, S. M., and A. C. Wilson, 1984 Molecular evolution in Drosophila and higher diptera. II. A time scale for fly evolution. J. Mol. Evol. **21:** 1–13.

Beye, M., 2004 The dice of fate: the csd gene and how its allelic composition regulates sexual development in the honey bee, Apis mellifera. BioEssays **26:** 1131–1139.

Beye, M., M. Hasselmann, M. K. Fondrk, R. E. Page and S. W. Omholt, 2003 The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell **114:** 419–429.

Boggs, R. T., P. Gregor, S. Idriss, J. M. Belote and M. McKeown, 1987 Regulation of sexual differentiation in *D. melanogaster* via alternative splicing of RNA from the *transformer* gene. Cell **50:** 739–747.

Burtis, K. C., and B. S. Baker, 1989 Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell **56:** 997–1010.

Butler, B., V. Pirrotta, I. Irminger-Finger and R. Nothiger, 1986 The sex-determining gene *tra* of Drosophila: molecular cloning and transformation studies. EMBO J. **5:** 3607–3613.

Cline, T. W., 1984 Autoregulation and functioning of a Drosophila gene product that establishes and maintains the sexually determined state. Genetics **107:** 231–277.

Cline, T. W., and B. J. Meyer, 1996 Vive la difference: males vs females in flies vs worms. Annu. Rev. Genet. **30:** 637–702.

Dauwalder, B., F. Amaya-Manzanares and W. Mattox, 1996 A human homologue of the *Drosophila* sex determination factor *transformer-2* has conserved splicing regulatory functions. Proc. Natl. Acad. Sci. USA **93:** 9004–9009.

Dübendorfer, A., M. Hediger, G. Burghardt and D. Bopp, 2002 *Musca domestica*, a window on the evolution of sex-determining mechanisms in insects. Int. J. Dev. Biol. **46:** 75–79.

Fujihara, T., M. Kawabe and K. Oishi, 1978 A sex-transformation gene in Drosophila melanogaster. J. Hered. **69:** 229–236.

Handler, A. M., and R. A. Harrell, 1999 Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Mol. Biol. **8:** 449–457.

Hediger, M., G. Burghardt, C. Siegenthaler, N. Buser, D. Hilfiker-Kleiner *et al.*, 2004 Sex determination in Drosophila melanogaster and Musca domestica converges at the level of the terminal regulator doublesex. Dev. Genes Evol. **214:** 29–42.

Hedley, M. L., and T. Maniatis, 1991 Sex-specific splicing and polyadenylation of *dsx* pre-mRNA requires a sequence that binds specifically to tra-2 protein in vitro. Cell **65:** 579–586.

Heinrichs, V., L. C. Ryner and B. S. Baker, 1998 Regulation of sex-specific selection of *fruitless* 5′ splice sites by *transformer* and *transformer-2*. Mol. Cell. Biol. **18:** 450–458.

Horn, C., and E. A. Wimmer, 2000 A versatile vector set for animal transgenesis. Dev. Genes Evol. **210:** 630–637.

Hoshijima, K., K. Inoue, I. Higuchi, H. Sakamoto and Y. Shimura, 1991 Control of *doublesex* alternative splicing by *transformer* and *transformer-2* in Drosophila. Science **252:** 833–836.

Inoue, K., K. Hoshijima, H. Sakamoto and Y. Shimura, 1990 Binding of the Drosophila *Sex-lethal* gene product to the alternative splice site of *transformer* primary transcript. Nature **344:** 461–463.

Inoue, K., K. Hoshijima, I. Higuchi, H. Sakamoto and Y. Shimura, 1992 Binding of Drosophila transformer and transformer-2 proteins to the regulatory elements of doublesex primary transcript for sex-specific RNA processing. Proc. Natl. Acad. Sci. USA **89:** 8092–8096.

Keyes, L. N., T. W. Cline and P. Schedl, 1992 The primary sex determination signal of Drosophila acts at the level of transcription. Cell **68:** 933–943.

Kuhn, S., V. Sievert and W. Traut, 2000 The sex-determining gene doublesex in the fly Megaselia scalaris: conserved structure and sex-specific splicing. Genome **43:** 1011–1020.

Kulathinal, R. J., L. Skwarek, R. A. Morton and R. Singh, 2003 Rapid evolution of the sex-determining gene, *transformer*: structural diversity and rate heterogeneity among sibling species of Drosophila. Mol. Biol. Evol. **20:** 441–452.

Marin, I., and B. S. Baker, 1998 The evolutionary dynamics of sex determination. Science **281:** 1990–1995.

McAllister, B. F., and G. A. T. McVean, 1999 Neutral evolution of the sex-determining gene *transformer* in Drosophila. Genetics **154:** 1711–1720.

McKeown, M., J. M. Belote and B. S. Baker, 1987 A molecular analysis of *transformer*, a gene in *Drosophila melanogaster* that controls female sexual differentiation. Cell **48:** 489–499.

McKeown, M., J. M. Belote and R. T. Boggs, 1988 Ectopic expression of the female *transformer* gene product leads to female differentiation of chromosomally male Drosophila. Cell **53:** 887–895.

Meise, M., D. Hilfiker-Kleiner, A. Dubendorfer, C. Brunner, R. Nothiger *et al.*, 1998 Sex-lethal, the master sex-determining gene in Drosophila, is not sex-specifically regulated in Musca domestica. Development **125:** 1487–1494.

Ohbayashi, F., M. G. Suzuki, K. Mita, K. Okano and T. Shimada, 2001 A homologue of the Drosophila doublesex gene is transcribed into sex-specific mRNA isoforms in the silkworm, Bombyx mori. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **128:** 145–158.

O'Neil, M. T., and J. M. Belote, 1992 Interspecific comparison of the *transformer* gene of Drosophila reveals an unusually high degree of evolutionary divergence. Genetics **131:** 113–128.

Pane, A., M. Salvemini, P. Delli Bovi, C. Polito and G. Saccone, 2002 The *transformer* gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. Development **129:** 3715–3725.

Raymond, C. S., C. E. Shamu, M. M. Shen, K. J. Seifert, B. Hirsch *et al.*, 1998 Evidence for evolutionary conservation of sex-determining genes. Nature **391:** 691–695.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella *et al.*, 1996 Control of male sexual behavior and sexual orientation in Drosophila by the *fruitless* gene. Cell **87:** 1079–1089.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of *Drosophila* with transposable element vectors. Science **218:** 348–353.

Saccone, G., I. Peluso, D. Artiaco, E. Giordano, D. Bopp *et al.*, 1998 The *Ceratitis capitata* homologue of the Drosophila sex-determining gene *Sex-lethal* is structurally conserved but not sex-specifically regulated. Development **125:** 1495–1500.

Saccone, G., A. Pane, G. Testa, M. Santoro, G. de Martino *et al.*, 2000 Sex determination in medfly: a molecular approach, pp. 491–496 in *Area-Wide Control of Fruitflies and Other Pest Insects*, edited by K.-H. Tan. Penerbit USM, Penang.

Saccone, G., A. Pane and L. C. Polito, 2002 Sex determination in flies, fruitflies and butterflies. Genetica **116:** 15–23.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.

Schutt, C., and R. Nothiger, 2000 Structure, function and evolution of sex determining systems in Dipteran insects. Development **127:** 667–677.

Seifert, H., E. V. Chen, M. So and F. Heffron, 1986 Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83:** 735–739.

Serna, E., E. Gorab, M. F. Ruiz, C. Goday, J. M. Erin-Lopez *et al.*, 2004 The gene *Sex-lethal* of the Sciaridae family (order Diptera, suborder Nematocera) and its phylogeny in dipteran insects. Genetics **168:** 907–921.

Shearman, D. C., and M. Frommer, 1998 The Bactrocera tryoni homologue of the Drosophila melanogaster sex-determination gene doublesex. Insect Mol. Biol. **7:** 355–366.

Sosnowski, B. A., J. M. Belote and M. McKeown, 1989 Sex-specific alternative splicing of RNA from the *transformer* gene results from sequence-dependent splice site blockage. Cell **58:** 449–459.

Tian, M., and T. Maniatis, 1993 A splicing enhancer complex controls alternative splicing of doublesex pre-mRNA. Cell **16:** 105–114.

Valcárcel, J., R. Singh, P. D. Zamore and M. R. Green, 1993 The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of *transformer* pre-mRNA. Nature **362:** 171–175.

Villella, A., D. A. Gailey, B. Berwald, S. Ohshima, P. T. Barnes *et al.*, 1997 Extended reproductive roles of the fruitless gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics **147:** 1107–1130.

Waterbury, J. A., J. I. Horabin, D. Bopp and P. Schedl, 2000 Sex determination in the Drosophila germline is dictated by the sexual identity of the surrounding soma. Genetics **155:** 1741–1756.

Wilkins, A. S., 1995 Moving up the hierarchy: a hypothesis on the evolution of a genetic sex determination pathway. BioEssays **17:** 71–77.

Communicating editor: T. C. Kaufman